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A Fragile X Mosaic Male With a Cryptic Full Mutation Detected in Epithelium But Not in Blood

Anne Maddalena, Karen N. Yadvish, W. Christine Spence, and Patricia N. Howard-Peebles

Genetics and IVF Institute, Fairfax (A.M., K.N.Y., W.C.S., P.N.H.-P.); Department of Human Genetics, Medical College of Virginia, Richmond, Virginia (A.M., P.N.H.-P.)

Individuals with developmental delay who are found to have only fragile X premutations present an interpretive dilemma. The presence of the premutation could be an unrelated coincidence, or it could be a sign of mosaicism involving a full mutation in other tissues. To investigate three cases of this type, buccal epithelium was collected on cytology brushes for Southern blot analysis. In one notable case, the blood specimen of a boy with developmental delay was found to have a premutation of 0.1 extra kb, which was shown by PCR to be an allele of 60 ± 3 repeats. There was no trace of a full mutation. Mosaicism was investigated as an explanation for his developmental delay, although the condition was confounded by prematurity and other factors. The cheek epithelium DNA was found to contain the premutation, plus a methylated full mutation with expansions of 0.9 and 1.5 extra kb. The three populations were nearly equal in frequency but the 1.5 kb expansion was the most prominent. Regardless of whether this patient has clinical signs of fragile X syndrome, he illustrates that there can be gross tissue-specific differences in molecular subpopulations in mosaic individuals. Because brain and epithelium are more closely related embryonically than are brain and blood, cryptic full mutations in affected individuals may be evident in epithelial cells while being absent or difficult to detect in blood. This phenomenon may explain some atypical cases of the fragile X phenotype associated with premutations or near-normal DNA findings. © 1996 Wiley-Liss, Inc.

KEY WORDS: fragile X, mosaic, epithelium, buccal, full mutation

INTRODUCTION

Typical individuals with fragile X syndrome have full mutations in the *FMR1* gene that are readily detectable in peripheral blood DNA. Full mutations are the expansions of six hundred to several thousand bases that occur in the region of CGG triplets located in the promoter region of this gene. The effect of the expansion is to prevent transcription of *FMR1* [Pieretti et al., 1991], as well as to make translation inefficient from any mRNA that might be transcribed from small unmethylated full mutations [Feng et al., 1995a]. The expansions of under 500 bp (premutations) found in unaffected carriers are not associated with suppressed transcription [Feng et al., 1995b].

Mosaicism in which both full mutations and premutations are present in the same individual is well documented in the affected population, and has been investigated as an indicator of phenotypic severity. Interlaboratory generalization has been difficult because the incidence of such patients ranges from 0–41% in various studies, and because the proportions of the different species are not usually reported [Nakahori et al., 1991; Rousseau et al., 1991; Macpherson et al., 1992; Knight et al., 1992; Yu et al., 1992; Snow et al., 1993; Rousseau et al., 1994; Nolin et al., 1994; Hagerman et al., 1994a]. The methodological or populational differences underlying this wide range are not clear, although some methods clearly enhance the detection of minor populations with small expansions. While the incidence of mosaic patients may be greater among higher functioning subjects [Hagerman et al., 1994a], mosaicism is not limited to this group so the prognostic implications are not clear-cut. A factor that may be influencing the prognostic correlation is tissue-to-tissue variation. Extreme tissue-specific differences in which one of the subpopulations is virtually absent in certain tissues could explain certain anomalous cases. For example, in very high functioning individuals with full mutations in blood cells the predominant species in the brain may be premutations. Evidence that cryptic premutations exist comes from studies showing that males

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Address reprint requests to Anne Maddalena, Genetics and IVF Institute, 3020 Javier Road, Fairfax, VA 22031.

with full mutations produce sperm with premutations [Reyniers et al., 1993], the report of an affected individual with full mutations in most tissues but premutations of different sizes in the testes and a lung tumor [de Graaf et al., 1995], and the report of an affected female with a full mutation in blood and mosaicism for a full mutation and a premutation in buccal epithelium [Taylor et al., 1994].

The converse type of hidden mosaicism also has been suspected in cases of affected individuals whose blood shows only a premutation or near-normal allele [Rousseau et al., 1991; Nakahori et al., 1991]. When a premutation is detected in an apparently affected patient, the finding must be considered a possible coincidence unless mosaicism for a full mutation can be proven. If a full mutation is present only at trace levels, or is so diffuse that it escapes notice, the PCR blot method may succeed in detecting its presence nonquantitatively [Pergolizzi et al, 1992]. Mueller et al. [1995] described such a case. If the full mutation is virtually not present in the blood, however, the only means of detecting it would be to test other tissues such as buccal epithelium. We have conducted such a search in four individuals in three families who have some feature(s) of fragile X syndrome but who appeared to have only premutations in leukocyte DNA. Our preliminary studies and the independent work of others showed that blood and cheek cells are generally consistent in terms of genotype (normal, premutation, or full mutation), although the smears or bands composing a full mutation usually vary distinctly between the two tissues [Taylor et al., 1994; Hagerman et al., 1994b]. We elected to use Southern analysis of unamplified DNA so that the actual proportions of any subpopulations would be evident. In three subjects the findings in cheek epithelium were identical or equivalent to the findings in blood, but in one notable case we discovered a cryptic full mutation that was not detectable in peripheral blood.

CLINICAL HISTORIES

Case 1 involved a mother-daughter pair. The child was a 5-year-old with hypotonia and developmental delay involving multiple parameters including fine motor control and language. Peripheral blood was submitted for routine fragile X testing. In addition to an allele of 20 ± 3 repeats, a nominal premutation of 58 ± 3 repeats was found. Blood from the patient's mother was subsequently tested and alleles of 23 and 53 (± 3) were found. The mother reported that she also had experienced language delay, which she "outgrew." For comparison, both members of this family participated in buccal cell testing.

Case 2 was a 28-year-old woman who had voluntary carrier testing. Alleles of 27 and 52 (± 3) repeats were found. During genetic counseling the subject offered the information that she has extreme difficulty with mathematics.

Case 3 was a 4-year-old boy on whom peripheral blood was submitted for fragile X testing with an indication of developmental delay. A premutation of 60 ± 3 repeats was found. In follow-up testing the patient's mother carried an upper allele of exactly the same size, as determined in side-by-side PCR reactions. Upon further inquiry the following history was communicated: The patient carries a diagnosis of cerebral palsy and developmental delay secondary to neonatal complications. He was delivered at 31 weeks with a tracheoesophageal fistula and required artificial ventilation for respiratory distress syndrome. He experienced intraventricular hemorrhage that resolved without shunting. Height and weight at 4 years were below the 5th centile, while head circumference was at the 50th centile. A systolic heart murmur was noted. His face is triangular with micrognathia and mild frontal bossing.

MATERIALS AND METHODS

DNA Isolation From Peripheral Blood by Salting Out

Blood was collected in EDTA, and a minimum of 24 hours later the nuclear pellet was isolated from a 3 ml aliquot. The pellet was suspended in 300 μl of an NTE solution containing 400 mM NaCl, 100 mM of Tris, pH 7.5, and 1 mM EDTA, and then rocked overnight at 56°C with 50 μl of 10 mg/ml proteinase K and 40 μl of 10% SDS. Thirty minutes after the addition of 40 μl of 6 M sodium perchlorate, 100 μl of saturated NaCl was added and the DNA was recovered from the supernatant after centrifugation. The DNA was concentrated by ethanol precipitation and dissolved in pH 7.5 Tris: EDTA (10 mM:1 mM).

DNA Isolation From Buccal Brushes by Phenol Extraction

Patients were asked to brush the inner surface of their cheeks with two or (optimally) four CytoSoft™ cytology brushes (Medical Packaging Corporation, Camarillo, CA), twirling the brushes to expose all surfaces. The brushes were immediately placed in a tube containing a lysis solution composed of 149 mM NH_4Cl, 2.6 mM EDTA, 0.7 mM KH_2PO_4, at a depth sufficient to submerge the bristle end of the brush. During transport, no effort was made to keep the brushes submerged. Upon arrival at the laboratory, liquid in the tube and any drops adhering to the brushes were collected and pooled. The solid material was pelleted and suspended in 300 μl of an NTE solution composed of 10 mM NaCl, 10 mM pH 7.5 Tris, and 10 mM EDTA, and then treated with proteinase K and SDS as above for 30 minutes at 56°C. After standard organic extraction using phenol: chloroform:isoamyl alcohol followed by chloroform:isoamyl alcohol, the DNA was concentrated by ethanol precipitation in the presence of 300 mM sodium acetate and dissolved in TE as above.

Southern Analysis

Three micrograms of DNA, or six micrograms for double loading, were treated with EcoRI and EagI as described by Rousseau et al. [1991]. Electrophoresis was performed in 20 cm gels of 0.8% agarose in TAE with ethidium bromide at 45–50 V. Duplicate samples were electrophoresed for 27–28 hours to resolve small expansions and for 5–6 hours to rule out full mutations [Levinson et al., 1994; Maddalena et al., 1994]. DNA was transferred to Sureblot (Oncor) or Biodyne B (Pall

Biosupport) membranes and probed with PCR-amplified copies of the insert of clone pStB12.3 [Oberlé et al., 1991], labeled by random priming. Expansions were estimated in increments of 50 bp, and if needed the sizes of high normal or small premutations were determined more precisely by PCR. Methylation at the EagI site was inferred to be present if bands were 5.2 kb or greater. X-inactivation in females was evaluated by densitometry of the normal active and enlarged active bands (Appraise Densitometry System, Beckman).

PCR Analysis

The method has been described in detail elsewhere [Levinson et al., 1994]. Essential aspects are the use of two rounds of PCR using nested primers, inclusion of 100% deazaGTP, and electrophoresis under denaturing conditions in sequencing gels. Although resolution at the level of 1–2 basepairs is often achieved, the results were reported with an error margin of 3 repeats.

RESULTS

Preliminary Normal and Full Mutation Controls

In normal controls, no extraneous bands were found in cheek DNA, as shown in Figure 1, lanes 6–9 and 11–14. In full mutation controls, no normal or premutated bands were found in cheek DNA from two affected males who had standard full mutations in peripheral blood (not shown). In both cases the full mutations were composed of distinct bands in the buccal DNA although they appeared as continuous smears in the blood DNA.

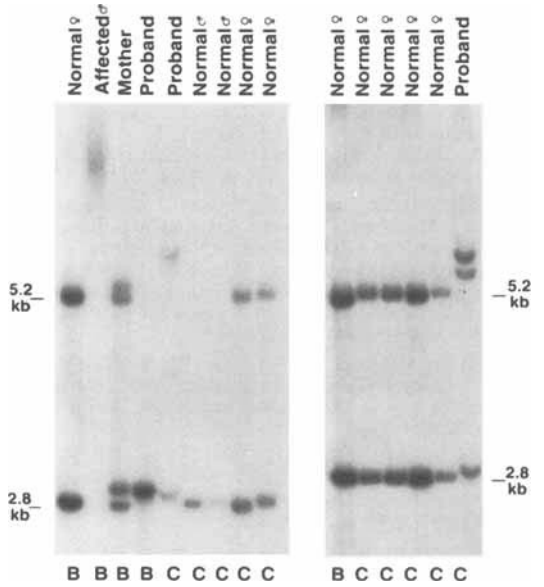

Fig. 1. EcoRI/EagI Southern blots of unamplified DNA from blood (B) or cheek (C) cells from Case 3 and controls, showing the lanes electrophoresed for 27–28 hours. The positions of the normal bands at 2.8 kb (active) and 5.2 kb (inactive) are indicated. Lanes 1 and 10 are normal female blood controls and lane 2 is a control full mutation from blood. Normal individuals in lanes 6–9 and 11–14 show that cheek DNA does not routinely contain extraneous bands. Blood DNA from the proband and his mother are shown in lanes 4 and 3, respectively. Cheek DNA from the proband is in lanes 5 and 15, with each lane containing DNA from two brushes processed in parallel but differing in DNA yield and digestability.

Case 1 and 2

The three females in these cases had upper alleles in the 50–60 repeat range. When buccal DNA specimens were compared to blood DNA by Southern analysis, the results were indistinguishable in size and no cryptic full mutations were observed (not shown). X-inactivation was noted to be distinctly different in each pair of samples, however, as shown in Table I. X-inactivation is worth noting as another tissue-to-tissue variable that could affect expression of a cryptic mosaic.

Case 3

Southern analysis of blood DNA from this developmentally delayed male revealed a premutation with no trace of a full mutation. His mother had a premutation of the same size as well as a normal length allele. These results are shown in lanes 3 and 4 of Figure 1. In several other gels (not shown), the result was the same, including double-loaded gels in which 5–6 hour electrophoresis was used to compress full mutation smears. Ethidium bromide staining of the gel showed that the patient's DNA was not degraded. To search for a full mutation in another tissue, four brushes of buccal epithelium were obtained from the patient, and separated for DNA extraction as two pairs. The entire DNA yield from each pair was digested and analyzed in one double-loaded gel. The longer migrating lanes are shown in Figure 1, lanes 5 and 15. Both aliquots of buccal DNA show the abundant presence of full mutations. The sample in lane 5 was lower in yield and also did not digest completely, as shown by the characteristic faint band at 4.1 kb, while the sample in lane 15 was high yield and fully digestible. The full mutation bands detected in lane 15 represent two discrete populations with expansions of 0.9 and 1.5 extra kilobases, and have the characteristic methylation at the EagI site. The intensities of the three bands, which are unbiased representations of the actual proportions, indicate that full mutations are the majority genotype in this tissue.

DISCUSSION

Practical Significance

The primary finding in this study is the presence of abundant copies of a methylated full mutation in epithelial cells of a patient whose peripheral blood DNA gave evidence of only a premutation. This changed his diagnosis from uncertain to positive for fragile X syndrome. The failure to detect a full mutation in the blood was not due to extreme diffuseness of the smear or to high background, but to the full mutation's extreme low level or absence. This type of mosaicism may be re-

TABLE I. The Percentage of Cells in Female Subjects in Which the Expanded Allele was Inactive, as Determined by Southern Blot Densitometry

Patient	Blood	Cheek
Case 1 daughter	72	51
Case 1 mother	22	~50[a]
Case 2	14	49

[a] Unable to be scanned.

sponsible for some of the anomalous mentally impaired patients with premutations or near-normal alleles that have been noted since DNA-based fragile X testing began. This small survey suggests that, among such patients, there may be a significant incidence of mosaic individuals with abundant but sequestered full mutations.

Genotype-Phenotype Correlation

In this particular case it is impossible to argue that the full mutation is responsible for this patient's developmental delay because of his complex history. Nevertheless, on embryological grounds it is logical to expect that if tissue-specific mosaicism exists, epithelial cells would be more likely than hematopoetic cells to share abnormalities with the nervous system. If other examples of this type can be identified, it will be interesting to see how well the ratio of full mutations to premutations in epithelial tissue correlates with clinical phenotype; it may be necessary to use genomic DNA blots rather than PCR blots to quantitate this effect. Another longstanding genotype-phenotype question that could be similarly studied is the role of lyonization in affected females; the females studied in cases 1 and 2 give evidence that X-inactivation patterns can vary dramatically between these two tissues. A third important question that could be investigated by direct Southern analysis of buccal epithelium is the clinical significance of incompletely methylated full mutations.

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